IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Wary and Humtsoe	§	**ART UNIT: 1644**
FILED: March 29, 2004	§	**EXAMINER: Haddad, M.M.**
SERIAL NO.: 10/812,238	§	
FOR: Uses of Vascular Endothelial Growth Factor and Type I Collagen Inducible Protein (VCIP)	§	**CONFIRMATION NO: 3362**
	§	**DOCKET: D6563**

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APPEAL BRIEF

This Appeal Brief is in furtherance of the Notice of Appeal transmitted via facsimile in this case on April 10, 2008. The fees required under 37 C.F.R. §41.20(b)(2) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF. However, if this is in error, please debit any additional fees due from Deposit Account No. 07-1185 on which Applicant's counsel is allowed to draw.

Respectfully submitted,

Date: Sept 10, 2008

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INDEX OF SUBJECT MATTER

I. REAL PARTY IN INTEREST

The real party in interest is The Texas A&M University System, as the owner of the entire right, title and interest in the patent application identified above, as evidenced by the Assignment recorded at Reel/Frame 015225/0079 on April 19, 2004.

II. RELATED APPEALS AND INTERFERENCES

Appellant is aware of no related appeals and/ interferences of the present invention

STATUS OF CLAIMS

Originally, claims 1-41 were filed and were subject to restriction in this Application. The withdrawn claims 1-7, 12-13, 18-19, 22-31, and 34-41 were canceled. Of the elected claims 8-11, 14-17, 20-21, and 32-33, claims 9-11, 16-17, 20-21, and 33 were canceled in response to Office Actions. Pending claims 8, 14-15 and 32, of which claims 8, 15 and 32 are independent, are the subject of this appeal.

IV. STATUS OF AMENDMENTS

Claims 10 and 16 were amended in response to a Restriction Requirement, submitted August 18, 2005. In response to an Office Action, mailed January 11, 2006, claims 8, 14-15, 20-21, and 32 were amended and claims 10-11, 16-17, and 33 were canceled. In response to the Final Office Action, submitted September 15, 2006, claims 8, 14-15, 20-21, and 32 were amended. In response to an Advisory Action, submitted November 30, 2006, claim 15 was amended and claims 20-21 were canceled. In response to an Office Action, submitted August 6, 2007, claim 9 was amended. In response to a Final Office Action, submitted February 11, 2008, claim 8 was amended and claim 9 was canceled. A Notice of Appeal was filed April 10, 2008 appealing the rejection of the pending claims 8, 14-15 and 32, as shown in Appendix A.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The subject matter of independent claim 8 is drawn to a method of inhibiting αvβ3 and/or α5β1 integrin ligand-mediated cell-cell interaction (pg. 20, ll. 14-15). The cells are contacted with an antibody directed against a peptide consisting of SEQ ID NO: 41 or consisting of SEQ ID No. 2 (pg. 20, ll. 14-16; pg. 21, ll. 25-26; Filg. 1L). These peptides are derived from a cell surface vascular endothelial growth factor and type I collagen inducible protein (VCIP) with a sequence consisting of SEQ ID No. 13 (pg. 20, ll. 14-16), that is, peptide sequences of SEQ ID NOS: 2 and 41 are contained with VCIP sequence of SEQ ID NO: 13 (pg. 19, ll. Fig. 1L). The antibody blocks binding of αvβ3 and/or α5β1 integrins to the cell surface VCIP which inhibits the αvβ3 and/or α5β1 integrin ligand-mediated cell-cell interaction (pg. 35-36, Example 16; Fig. 9).

The subject matter of independent claim 15 is drawn to a method of inhibiting tumor growth, inflammation and/or angiogenesis in a patient (pg. 20, ll. 14-15). The patient is administered an antibody directed against a peptide consisting of SEQ ID NO: 41 or consisting of SEQ ID No. 2 (pg. 20, ll. 14-16; pg. 21, ll. 25-26; Filg. 1L). These peptides are derived from a cell surface vascular endothelial growth factor and type I collagen inducible protein (VCIP) with a sequence consisting of SEQ ID No. 13 (pg. 20, ll. 14-16), that is, peptide sequences of SEQ ID NOS: 2 and 41 are contained with VCIP sequence of SEQ ID NO: 13 (pg. 19, ll. Fig. 1L). The antibody blocks binding of αvβ3 and/or α5β1 integrins to the cell surface VCIP (pg. 35-36, Example 16; Fig. 9) which inhibits

tumor growth, inflammation and/or angiogenesis in the patient (pg. 35-36, Example 16; Fig. 9).

The subject matter of independent claim 32 is drawn to a method of inhibiting angiogenesis and the formation of capillaries in a patient in need of such a treatment (pg. 20, ll. 14-15). The patient is administered a pharmacologically effective amount of an antibody directed against a peptide consisting of SEQ ID NO: 41 or consisting of SEQ ID No. 2 (pg. 20, ll. 14-16; pg. 21, ll. 25-26; Filg. 1L). These peptides are derived from a cell surface vascular endothelial growth factor and type I collagen inducible protein (VCIP) with a sequence consisting of SEQ ID No. 13 (pg. 20, ll. 14-16), that is, peptide sequences of SEQ ID NOS: 2 and 41 are contained with VCIP sequence of SEQ ID NO: 13 (pg. 19, ll. Fig. 1L). The antibody inhibits $\alpha v\beta 3$ and/or $\alpha 5\beta 1$ integrin-mediated cell-cell interaction which inhibits angiogenesis and the formation of capillaries in the patient in need of such a treatment (pg. 51, ll. 8-10; Fig. 22).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Whether claims 8 and 14-15 are anticipated by **Vassilev** *et al.*, *Blood*, 93(11): 3624-3631, Jun 1, 1999,) as is evidenced by **Bendayan** (*J Histochem Cytochem*, 43: 881-886, 2000) under 35 U.S.C. §102(b).

Whether claims 15 and 32 are obvious over **U.S. Patent No. 5,807,819** in view of U.S. Patent No. 5,567,440) and **Vassilev** *et al.* (*Blood*, 93(11): 3624-3631, Jun 1, 1999,) as is evidenced by **Bendayan** (*J Histochem Cytochem*, 43: 881-886, 2000) under 35 U.S.C. §103(a).

VII. ARGUMENT

Rejection of Claims 8 and 15 under 35 U.S.C. §102(b) over Vassilev *et al.*, as is evidenced by Bendayan

It is well established that in order to anticipate a claim under 35 U.S.C. §102(b), each and every element of the claim should be described in a single prior art reference, either expressly or inherently. Importantly, the identical invention must be shown in as complete detail as is contained in the instant invention. Applicant's claim 8 is directed towards a method of inhibiting cell-cell interaction by blocking the interaction between VCIP and the αvβ3 and α5β1 integrins via contact with an antibody specifically directed against a peptide consisting of SEQ ID NO: 41 or consisting of SEQ ID No. 2. Independent claim 15 is drawn to method of inhibiting tumor growth, inflammation and/or angiogenesis in a patient by administering an antibody specifically antibody directed against a peptide consisting of SEQ ID NO: 41 or consisting of SEQ ID No. 2 to block the interaction between VCIP and the αvβ3 and α5β1 integrins.

Applicants respectfully submit that **Vassilev** *et al.* do not teach a method of inhibiting αvβ3 and/or α5β1 integrin ligand-mediated cell-cell interaction, as recited in Applicants' claim 8. **Vassilev** *et al.* do not teach a method of inhibiting tumor growth, inflammation and/or angiogenesis in a patient, as recited in Applicants' claim 15. Applicants demonstrate that Applicants' antibody blocks the interaction between VCIP and the αvβ3 and α5β1 integrins (pg. 35-36, Example 16;

Fig. 9). Also, Applicants teach that αvβ3 and/or α5β1 integrin ligand-mediated cell-cell interaction have clear pathological consequences, such as inflammation and tumor-induced angiogenesis (page 19, ll. 18-24). In addition, Applicants demonstrate in an in vivo mouse model of human colon cancer that VCIP is expressed in tumor vasculatures near αvβ3, potentiates tumor growth and regulates tumor angiogenesis by recruiting endothelial cells (page 48, Example 27; Figures 20A-20).

In distinct contrast, **Vassilev** *et al.* teach a method of inhibiting adenosine diphosphate-induced platelet aggregation (pg. 3624, 2nd col., 2nd full PP) by naturally produced antibodies eluted from a pool of intravenous immunoglobulin (IVIg) obtained from several thousand healthy donors (pg. 3624, 1st col., 1st PP). The antibodies in the eluate bind to a synthetic RGD sequence-containing peptide AVTGRGDSPA (pg. 3624, 2nd col., last PP). Applicants submit that inhibiting platelet aggregation by a pool of naturally occurring antibodies is not the equivalent to inhibiting αvβ3 and/or α5β1 integrin ligand-mediated cell-cell interaction nor the same as inhibiting tumor growth, inflammation and/or angiogenesis in a patient, as recited in Applicants' claims 8 and 15.

Second, **Vassilev** *et al.* do not teach blocking the interaction between VCIP and the αvβ3 and α5β1 integrins. Also, **Vassilev** *et al.* do not teach VCIP or αvβ3 and α5β1 integrins. As stated supra by Applicants, **Vassilev** *et al.* disclose inhibiting aggregation of platelets.

Third, **Vassilev** *et al.* do not teach Applicants' SEQ ID NOS: 2 and 41 nor does **Vassilev** *et al.* teach antibodies specifically directed against these sequences. Also, as stated supra, **Vassilev** *et al.* do not teach VCIP nor the VCIP sequence of SEQ ID NO: 13. Applicants' antibody is directed against specific peptide sequences of SEQ ID NO: 2 and 14 that are contained within a specific VCIP sequence. Except for the RGD amino acids, Applicants' SEQ ID NOS: 2 and 41 are neither identical nor homologous to the AVTGRGDSPA peptide disclosed in **Vassilev** *et al.*

Vassilev *et al.* teach that the eluted antibodies bind to an RGD-motif containing peptide AVTGRGDSPA (pg. 3624, 2nd col., last PP). **Vasilev** *et al.* demonstrate that their antibody eluate can bind to fibronectin, fibrinogen, vitronectin, VWF and laminin in a (pg. 3626, col. 1, 1st full PP). Just because an antibody, such as the antibodies in **Vassilev** *et al.*, bind to an RGD motif-containing peptide, does not inherently mean the antibodies would necessarily have any inhibitory action upon binding, absent evidence to the contrary.

The Examiner contends that the antibodies in **Vassilev** *et al.* would bind to the peptide of SEQ ID NO: 2 and 41 due to the shared sequence homology of the RGD motif. In distinct contrast to the Examiner's assertion, Applicants demonstrated that Applicants' antibody did not react with other RGD-containing extracellular matrix molecules such as fibronectin, vitronectin, or type I collagen (pg. 40, ll. 3-4). The Examiner relies on **Bendayan** who the Examiner states teaches cross-reactivity of antibodies. Applicants submit that **Bendayan** characterizes the specific reactivity of a monoclonal antibody produced to human proinsulin.

Bendayan shows that although the antibody is highly specific, it is nevertheless able to bind to not only human proinsulin, but to proinsulin from other species and to glucagon, based upon conservation of an Arg-Arg dipeptide sequence in each of these molecules. **Bendayan** concludes that an antibody directed against a small peptide sequence, could bind both a specific antigen and molecules **not related** (Applicants emphasis) to the original antigen and could possibly yield a false positive immunocytochemical result (pg. 886, last PP).

Applicants demonstrate that Applicants' antibody did not crossreact with mouse antigens (pg. 40, ll. 5). Applicants submit that the Examiner has not offered any evidence that the antibodies in **Vassilev** *et al.* will bind an RGD motif in VCIP that results in blocking an interaction with $\alpha v\beta 3$ and/or $\alpha 5\beta 1$ integrins (claim 8) or in inhibiting tumor growth, inflammation and/or angiogenesis in a patient (claim 15). Therefore, Applicants submit that the Examiner is rejecting claims 8 and 15 over **Vassilev** *et al.* based on only a mere possibility that the antibodies taught therein would produce an inhibitory or blocking effect on VCIP interaction with $\alpha v\beta 3$ and/or $\alpha 5\beta 1$ integrins.

Also, Applicants submit that Applicants' antibody is specifically directed against all the amino acids in Applicants' peptides of SEQ ID NOS: 2 and 41, and not merely the RGD motif. In partial support for this argument, Applicants previously submitted **Pedchenko** *et al.* who taught that $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins bind both the proximal RGD site and non-RGD motifs within the noncollagenous domain of the alpha3 chain of Type IV collagen. Thus, **Pedchenko** *et al.* teach that

both the RGD and non-RGD motifs contribute to the mechanism of endothelial cell adhesion in the human vasculature.

For these reasons, Applicants submit that claims 8 and 15 are not anticipated under 35 U.S.C. §102(b) by **Vassilev** *et al.* as evidenced by **Bendayan**. Accordingly, Applicants respectfully request the Board of Patent Appeals and Interferences to reverse the rejection of claims 8 and 14-15 under 35 U.S.C. §102(b).

Rejection of Claims 15 and 32 under 35 U.S.C. §103(a) over U.S. Patent No. 5,807,819 in view of U.S. Patent No. 5,567,440 and Vassilev *et al.*, as is evidenced by Bendayan

As stated supra, independent claim 15 is drawn to a method of inhibiting tumor growth, inflammation and/or angiogenesis in a patient by administering an antibody directed against a peptide consisting of SEQ ID No. 41 or consisting of SEQ ID No. 2 that is derived from a cell surface vascular endothelial growth factor and type I collagen inducible protein (VCIP) consisting of SEQ ID No. 13 to block the interaction between VCIP and the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins. Applicants demonstrate that Applicants' antibody blocks the interaction between VCIP and the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins (pg. 35-36, Example 16; Fig. 9). Also, Applicants teach that $\alpha v\beta 3$ and/or $\alpha 5\beta 1$ integrin ligand-mediated cell-cell interaction have clear pathological consequences, such as inflammation and tumor-induced angiogenesis (pg. 19, ll. 18-24). In addition, Applicants demonstrate in an in vivo mouse model of human colon cancer that VCIP is

expressed in tumor vasculatures near αvβ3, potentiates tumor growth and regulates tumor angiogenesis by recruiting endothelial cells (pg. 48, Example 27; Figs. 20A-20).

Independent claim 32 is drawn to a method of inhibiting angiogenesis and capillary formation in a patient by administering directed against a peptide consisting of SEQ ID No. 41 or consisting of SEQ ID No. 2 that is derived from a cell surface vascular endothelial growth factor and type I collagen inducible protein consisting of SEQ ID No. 13 to inhibit αvβ3 and/or α5β1 integrin-mediated cell-cell interaction. Applicants demonstrate that Applicants' antibody blocks angiogenesis and capillary formation in vitro. Applicants antibody inhibited the formation of new capillaries and reduced the number of capillaries by about 45% to 55% in endothelial cells (pg. 51, ll. 8-10; Fig. 22).

A determination of obviousness requires, *inter alia*, that all the claim elements must be known in the art. Most importantly, Applicants' teach that prior to the instant invention, VCIP was not known to function as an integrin ligand and had no known function other than lipid phosphatase activity (pg. 18, ll. 28 to pg. 19, ll. 3; Abstract). Thus, as this function of VCIP was unknown at the time of the instant invention, Applicants submit that the a person having ordinary skill in the art, after consideration of U.S. Patent No. 5,807,819 with U.S. Patent No. 5,567,440 and Vassilev *et al.* would not have had a reasonable expectation that Applicant's method of inhibiting tumor growth, inflammation and/or angiogenesis in a patient by administering an antibody directed against a peptide consisting of SEQ ID No. 41 or consisting of SEQ ID No. 2 that is derived from a cell surface vascular

endothelial growth factor and type I collagen inducible protein (VCIP) consisting of SEQ ID No. 13 to block the interaction between VCIP and the αvβ3 and α5β1 integrins would be successful.

One could assume, *arguendo*, that one of ordinary skill in the art could substitute the RGD-motif containing CRGDDVC cyclic peptide taught by U.S. Patent No. 5,807,819 (see SEQ ID NO: 17) with the antibodies that bind the RDG-motif containing peptide AVTGRGDSPA taught by **Vassilev** *et al.* (as discussed supra) because U.S. Patent 5,567,440 discloses that cell adhesion interactions have a role in human disease and that these interactions can be interrupted by competitive inhibition e.g., with antibodies, soluble ligands which act as a receptor antagonist such as cyclic RGD peptides, soluble receptors or other competitors (col. 1, ll. 17-30). However, simply making this combination cannot render claims 15 and 32 obvious without one of ordinary skill in the art being able to predict that VCIP would function as an integrin ligand.

The Examiner states that the claim limitation "blocks the binding of integrins to cell surface VCIP" would be an expected property of the resultant method based on the cited combination. The Examiner's statement notwithstanding, Applicants submit that one of ordinary skill in the art in making the cited combination could not have predicted with a reasonable expectation of success that the antibodies in **Vassilev** *et al.* would block the interaction between VCIP and the αvβ3 and α5β1 integrins (claim 15) or inhibit αvβ3 and/or α5β1 integrin-mediated cell-cell interaction (claim 32) absent the disclosure in Applicants' specification.

Secondly, a determination of obviousness requires a reasonable expectation of success. The scope of both claims 15 and 32 encompass administering an antibody directed against a peptide consisting of SEQ ID No. 41 or consisting of SEQ ID No. 2 that is derived from a cell surface vascular endothelial growth factor and type I collagen inducible protein (VCIP) consisting of SEQ ID No. 13. The resultant antibody, upon administration to a patient, blocks the interaction between VCIP and the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins (pg. 35-36, Example 16; Fig. 9). Applicants respectfully reiterate that the present invention is the first to disclose that VCIP-derived peptides and proteins act as integrin ligands. As this claim element was unknown, a person having ordinary skill in this art would not have had a reasonable expectation of success based on the cited combination of references.

Accordingly, Applicants respectfully request that the Board of Patent Appeals and Interferences reverses the rejection of claims 15 and 32 under 35 U.S.C. §103(a).

Respectfully submitted,

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VIII. CLAIMS APPENDIX

Claim 8. A method of inhibiting αvβ3 and/or α5β1 integrin ligand-mediated cell-cell interaction, comprising:

contacting the cells with an antibody directed against a peptide consisting of SEQ ID NO: 41 or consisting of SEQ ID No. 2 that is derived from a cell surface vascular endothelial growth factor and type I collagen inducible protein (VCIP) consisting of SEQ ID No. 13, wherein said contact with the antibody blocks binding of αvβ3 and/or α5β1 integrins to the cell surface vascular endothelial growth factor and type I collagen inducible protein (VCIP), thereby inhibiting the αvβ3 and/or α5β1 integrain ligand-mediated cell-cell interaction.

Claim 14. The method of claim 8, wherein said cell-cell interaction contributes to inflammation or angiogenesis.

Claim 15. A method of inhibiting tumor growth, inflammation and/or angiogenesis in a patient, comprising:

administering to said patient an antibody directed against a peptide consisting of SEQ ID No. 41 or consisting of SEQ ID No. 2 that is derived from a cell surface vascular endothelial growth factor and type I collagen inducible protein (VCIP) consisting of SEQ ID No. 13, wherein said antibody blocks binding of αvβ3 and/or α5β1 integrins to the cell surface vascular endothelial growth factor and type

I collagen inducible protein (VCIP), thereby inhibiting tumor growth, inflammation and/or angiogenesis in the patient.

Claim 32. A method of inhibiting angiogenesis and the formation of capillaries in a patient in need of such a treatment, comprising:

administering to said patient a pharmacologically effective amount of an antibody directed against a peptide consisting of SEQ ID No. 41 or consisting of SEQ ID No. 2 that is derived from vascular endothelial growth factor and type I collagen inducible protein (VCIP) consisting of SEQ ID No. 13, wherein said antibody inhibits $\alpha v\beta 3$ and/or $\alpha 5\beta 1$ integrin-mediated cell-cell interaction, thereby inhibiting angiogenesis and the formation of capillaries in the patient in need of such a treatment.

IX. EVIDENCE APPENDIX

Pedechenko *et al.* J Biol Chem., 279(4): 2772-2780 (2004) was entered into the record after filing the response to Final Office Action, submitted February 11, 2008.

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$\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrins Bind Both the Proximal RGD Site and Non-RGD Motifs within Noncollagenous (NC1) Domain of the $\alpha3$ Chain of Type IV Collagen

IMPLICATION FOR THE MECHANISM OF ENDOTHELIAL CELL ADHESION*

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The NC1 domains of human type IV collagen, in particular $\alpha3$NC1, are inhibitors of angiogenesis and tumor growth (Petitclerc, E., Boutaud, A., Prestayko, A., Xu, J., Sado, Y., Ninomiya, Y., Sarras, M. P., Jr., Hudson, B. G., and Brooks, P. C. (2000) *J. Biol. Chem.* 275, 8051–8061). The recombinant $\alpha3$NC1 domain contained a RGD site as part of a short collagenous sequence at the N terminus, designated herein as RGD-$\alpha3$NC1. Others, using synthetic peptides, have concluded that this RGD site is nonfunctional in cell adhesion, and therefore, the antiangiogenic activity is attributed exclusively to $\alpha_v\beta_3$ integrin interactions with non-RGD motifs of the RGD-$\alpha3$NC1 domain (Maeshima, Y., Colorado, P. C., and Kalluri, R. (2000) *J. Biol. Chem.* 275, 23745–23750). This nonfunctionality is surprising given that RGD is a binding site for $\alpha_v\beta_3$ integrin in several proteins. In the present study, we used the $\alpha3$NC1 domain with or without the RGD site, expressed in HEK 293 cells for native conformation, as an alternative approach to synthetic peptides to assess the functionality of the RGD site and non-RGD motifs. Our results demonstrate a predominant role of the RGD site for endothelial adhesion and for binding of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Moreover, we demonstrate that the two non-RGD peptides, previously identified as the $\alpha_v\beta_3$ integrin-binding sites of the $\alpha3$NC1 domain, are 10-fold less potent in competing for integrin binding than the native protein, indicating the importance of additional structural and/or conformational features of the $\alpha3$NC1 domain for integrin binding. Therefore, the RGD site, in addition to non-RGD motifs, may contribute to the mechanisms of endothelial cell adhesion in the human vasculature and the antiangiogenic activity of the RGD-$\alpha3$NC1 domain.

Type IV collagen is the major constituent of basement membranes, a specialized form of extracellular matrix underlying all epithelia, that compartmentalizes tissues and provides molecular signals for influencing cell behavior. The type IV collagen family is comprised of six α-chains ($\alpha1$–$\alpha6$) that assemble into three kinds of triple-helical protomers of different chain composition. Each protomer has three functional domains: a 7 S domain at the N terminus, a long triple-helical collagenous domain in the middle of the molecule, and a trimeric noncollagenous (NC1) domain at the C terminus. Protomers self-assemble into networks by end-to-end associations that connect four 7 S domains at one end and connect two NC1 trimeric domains at the other end, forming an NC1 hexamer configuration (1). Three types of networks are known: an $\alpha1{\cdot}\alpha1{\cdot}\alpha2$ network, present in the basement membranes of all tissues and animal phyla and $\alpha3{\cdot}\alpha4{\cdot}\alpha5$ and $\alpha1{\cdot}\alpha2{\cdot}\alpha5.\alpha6$ networks that have a restricted tissue distribution. These networks are essential for tissue development and function. They provide mechanical stability, a scaffold for assembly of other macromolecular components, and act as a ligand for integrins, receptors that mediate cell adhesion, migration, growth, and differentiation.

Cell adhesion to the ubiquitous $\alpha1{\cdot}\alpha1{\cdot}\alpha2$ (IV) network has been demonstrated for a variety of cell types (2–4), including endothelial (5, 6) and tumor cell lines (7, 8). It is mediated by integrin binding to both triple-helical and NC1 domains. Specifically, integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ were identified as major receptors for the collagenous domain (9), and their binding sites have been subsequently mapped (10, 11). Additional integrins, such as $\alpha_3\beta_1$, that bind the triple-helical domain may be involved (12, 13). The NC1 domain was initially characterized as a ligand for $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins in human mesangial cells (4), and binding of $\alpha_1\beta_1$ integrin to recombinant $\alpha1$NC1 was later confirmed (14). In contrast, recombinant $\alpha2$NC1 was identified as a novel ligand for a different subset of integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_3\beta_1$) in endothelial cells, suggesting the existence of a non-RGD-binding motif (15).

Cell adhesion to the $\alpha3{\cdot}\alpha4{\cdot}\alpha5$(IV) network is less understood because only the recombinant NC1 domains are available for study. It is interesting that the individual human NC1 domains, expressed in mammalian cells, have strikingly different effects on endothelial cells. The $\alpha3$NC1 domain strongly activates both adhesion and migration, whereas the $\alpha4$NC1 and $\alpha5$NC1 domains are inactive (15), despite high sequence homology among all three NC1 domains, suggesting that the $\alpha3$NC1 domain contains unique structural determinants mediating these effects. Experiments with neutralizing antibodies provided the first evidence that endothelial cell adhesion to $\alpha3$NC1 domain was mediated by $\alpha_v\beta_3$ integrin (15). In these studies, the recombinant protein contained a RGD site within a 12-residue collagenous sequence proximal to the $\alpha3$NC1 domain.

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In this report this recombinant protein is designated RGD-α3NC1 to emphasize the presence of the RGD site. Cell adhesion to the RGD-α3NC1 domain could be mediated by $\alpha_v\beta_3$ binding to the RGD sequence, a well known integrin-binding site in numerous proteins, or to non-RGD motifs within the α3NC1 domain. In a subsequent study, Maeshima *et al.* (16) showed that this RGD site of the RGD-α3NC1 domain, termed tumstatin, was nonfunctional in cell adhesion and concluded that it does not bind $\alpha_v\beta_3$ integrin. Instead, they identified a non-RGD region comprising residues 54–132 of the α3NC1 domain that bound the $\alpha_v\beta_3$ integrin, which was later narrowed down to 25 residues using deletion mutagenesis and synthetic peptides (17). Another non-RGD region of the α3NC1 domain, residues 185–203, identified by Han *et al.* (18) was demonstrated to inhibit proliferation of melanoma cells, and the receptor for this synthetic peptide was identified as $\alpha_v\beta_3$ integrin by affinity chromatography (19). Whether these two non-RGD motifs quantitatively account for the adhesive activity of the native RGD-α3NC1 domain and its capacity to bind $\alpha_v\beta_3$ integrin has not been addressed.

The NC1 domains of certain α-chains of type IV collagen also display activity as inhibitors of angiogenesis and tumor growth. The capacity of the exogenous α1NC1 and α2NC1 domains to disrupt basement membrane assembly, blocking tissue development *in vivo*, was first described in *Hydra vulgaris* (20). This observation led us to evaluate the capacity of individual recombinant NC1 domains to perturb the basement membrane assembly of developing blood vessels. The α2NC1, RGD-α3NC1, and α6NC1 domains potently inhibited both angiogenesis and tumor growth in a chick chorioallantoic membrane system, with RGD-α3NC1 exhibiting the strongest effect, whereas NC1 domains of the α1, α4, and α5 chains had no effect. This inhibitory activity is presumably mediated by the $\alpha_v\beta_3$ integrin binding to the RGD and/or non-RGD motifs (15). Subsequent studies have revealed that the anti-angiogenic activity of RGD-α3NC1 domain (tumstatin) is potentially associated with inhibition of cell proliferation, induction of apoptosis, and activation of caspase-3 specifically in endothelial cells (21). Furthermore, it has been shown that both tumstatin and its non-RGD peptide inhibit cap-dependent translation only in endothelial cells through negative regulation of mTOR signaling (22, 23), implicating that the anti-angiogenic activity depends on binding of the $\alpha_v\beta_3$ integrin to non-RGD motifs but not to the RGD. More recent studies lead to the supposition that the α3NC1 domain can function as an endogenous suppressor of $\alpha_v\beta_3$ integrin-mediated pathologic angiogenesis and tumor growth (24). The finding that the RGD site is nonfunctional for $\alpha_v\beta_3$ integrin binding, revealed with synthetic peptides, is surprising, because it is a key binding site in several matrix proteins (25, 26).

An understanding of the molecular mechanism of integrin-mediated cell adhesion of the RGD-α3NC1 domain is ultimately important given the potential role of this protein as a pharmacological and endogenous regulator of angiogenesis and tumor growth. This requires identification of the integrin receptors, recognition sites within RGD-α3NC1 domain, and the ligand contact points within the integrin. In the present study, an alternative to the synthetic peptides approach was used to assess the functionality of the RGD and non-RGD motifs in the context of native protein conformation. This was accomplished by using recombinant proteins/chimeras with and without the RGD sequence, expressed in mammalian cells to ensure native conformation, for endothelial cell adhesion and integrin binding assays. Our results provide unambiguous evidence that both the RGD and non-RGD motifs bind $\alpha_v\beta_3$ integrin and mediate the adhesion of endothelial cells to the RGD-α3NC1 domain. These findings suggest that both motifs may contribute to the anti-angiogenic activity of the RGD-α3NC1 domain.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies Mab-3 to α3NC1 were purchased from Wieslab AB (Lund, Sweden). Goodpasture autoantibodies were purified from GP[1] human serum by affinity chromatography on protein A-agarose. Purified $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_1\beta_1$, and $\alpha_5\beta_1$ integrins and monoclonal integrin antibodies LM609 (anti-$\alpha_v\beta_3$) and P1F6 (anti-$\alpha_v\beta_5$) were from Chemicon (Temecula, CA). Integrin monoclonal antibodies P2W7 (anti-α_v) and 4B7R (anti-β_1) were from Santa Cruz (Santa Cruz, CA); monoclonal antibodies AIIB2 (anti-β_1) and BIIG2 (anti-α_5), developed by Dr. Caroline H. Damsky, were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). M2 monoclonal antibodies to FLAG peptide, RGDS peptide, and fibronectin were from Sigma; vitronectin was from TaKaRa Biomedicals (Shiga, Japan). Synthetic peptides T3 (LQRFTTMPFLFCNVNDVCNF) and 185–203 (CNYYSNSYSFWLASLNPER) were purchased from Multiple Peptide Synthesis (San Diego, CA) and SynPep Corp. (Dublin, CA), respectively.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were obtained from BioWhittaker (Charlotte, NC). The cells were grown in EGM-2 MV medium (BioWhittaker) and used between passages 3 and 7.

Proteins—Recombinant human NC1 domains of type IV collagen that carried the FLAG sequence on the N terminus were stably expressed in HEK 293 cells and purified from conditioned medium by affinity chromatography on anti-FLAG agarose as described previously (27). α3NC1 domain was created as a deletion mutant of RGD-α3NC1 lacking 12 amino acid residues from the N terminus by PCR using RGD-α3/pRC/CMV expression vector as a template and the following primers: 5′-ATA TGC TAG CTG CAA CCT GGA CAA CGA GAG (forward) and 5′-CAG CGA GCT CTA GCA TTT AGG (reverse). Purified PCR product was digested with NheI and ApaI restriction enzymes and subcloned into the pRc/CMV vector for protein expression. Prior to transfection in HEK 293 cells, the α3NC1 insert was sequenced in both directions to verify the sequence.

Cell Adhesion Assay—Proteins in TBS buffer or synthetic peptides in 50 mM Na_2CO_3/$NaHCO_3$ buffer, pH 9.5, were immobilized on 96-well plates (Nunc, Rochester, NY) at 4 °C overnight. Nonspecific binding sites were blocked with 1% BSA in TBS for 2 h at 30 °C, and the wells were washed twice with TBS. Subconfluent HUVEC were harvested, washed, and resuspended in adhesion buffer containing Ham's F-12/Dulbecco's modified Eagle's medium, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.5% BSA. 5×10^4 cells were added to each well and allowed to attach for 60 min at 37 °C in a CO_2 incubator. In some experiments, the cells were pretreated for 30 min with integrin-specific antibodies or peptides prior to their addition to the wells. After removal of the nonattached cells by washing with TBS, the attached cells were fixed and stained with 0.1% crystal violet as described (28). The wells were washed three times with TBS, and cell-associated crystal violet was eluted by the addition of 100 μl of 10% acetic acid. Cell adhesion was quantified by measuring the absorbance of eluted dye at 595 nm with a microtiter plate reader. All of the presented data were corrected for background binding in blank wells blocked with BSA.

Cell Membrane Labeling—HUVEC were grown in EGM-2 MV medium, detached from culture dishes with 2 mM EDTA in Hanks' balanced salt solution, and collected by centrifugation for 5 min at 800 × *g*. After two washes with cold phosphate-buffered saline, the cells were resuspended in phosphate-buffered saline at 1×10^7 cells/ml. Sulfo-NHS-biotin (Pierce) was added to a final concentration of 100 μg/ml and incubated with cells for 1 h at room temperature with gentle mixing. The cells (1×10^8) were washed three times with cold phosphate-buffered saline, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM phenylmethylsulfonyl fluoride and extracted for 30 min at 4 °C with TBS containing 100 mM octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). The supernatant was collected after centrifugation for 30 min at 16,000 × *g* and stored at −70 °C.

Affinity Chromatography—Purified recombinant RGD-α3NC1 (1 mg/ml of resin) was coupled to the Affi-Gel 10 (Bio-Rad) in 0.1 M MOPS buffer, pH 7.0. The remaining active groups were blocked with 0.1 M diethanolamine. Coupling efficiency was 75% as determined by absorb-

[1] The abbreviations used are: GP, Goodpasture; BSA, bovine serum albumin; HUVEC, human umbilical vein endothelial cells; TBS, Tris-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; NC1, the noncollagenous domain.

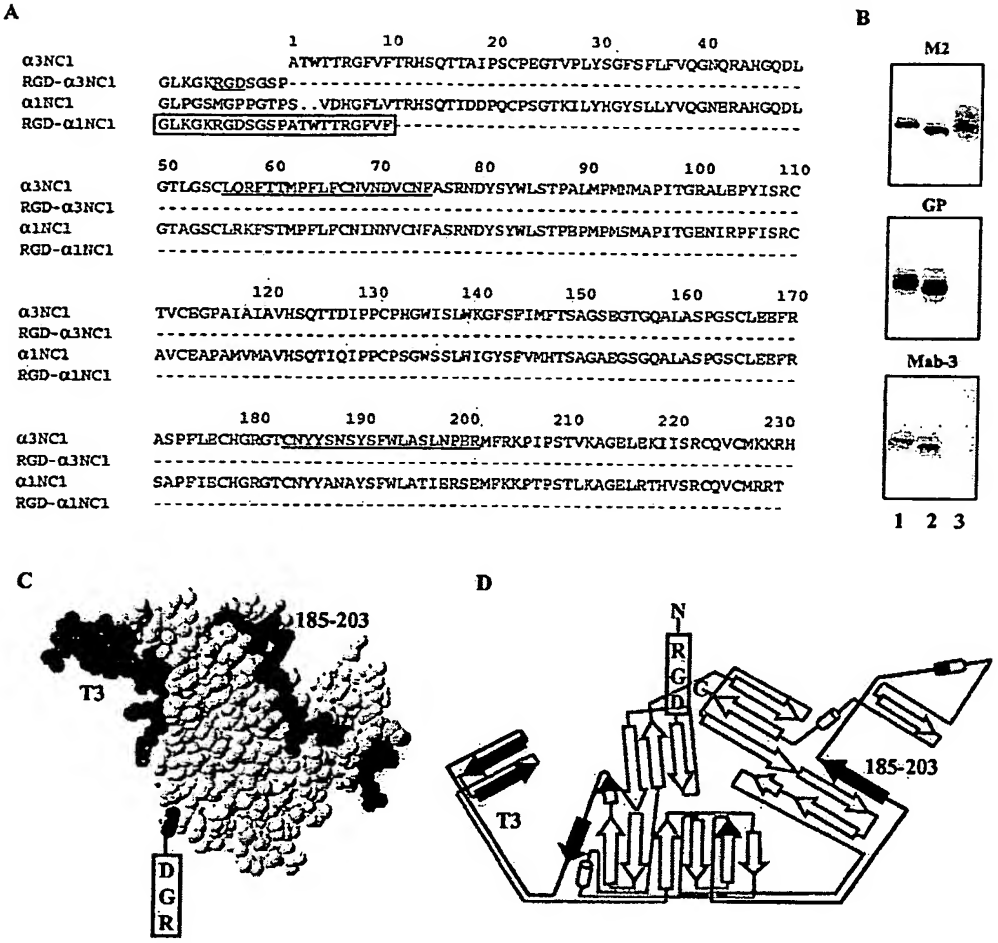

FIG. 1. *A*, aligned amino acid sequence of recombinant NC1 domains. Amino acid residues in the RGD-α3 identical to α3NC1 and in RGD-α1 identical to those in α1NC1 are represented by *dashes*. RGD site and sequences corresponding to synthetic peptides T3 (56–75) and 185–203 in α3NC1 are *underlined*. The 22 N-terminal amino acids substituted for RGD-α3 in RGD-α1 (formerly published as chimera C7 (30)) are *boxed*. *B*, Western immunoblotting of recombinant NC1 domains with anti-FLAG (M2), GP and Mab-3 antibodies. *Lane 1*, RGD-α3NC1; *lane 2*, α3NC1; *lane 3*, RGD-α1NC1. *C* and *D*, space-filling model (*C*) and topology diagram (*D*) of RGD-α3NC1 based on the published crystal structure of α1NC1 domain from NC1 hexamer (44). The positions of the RGD, T3, and 185–203 sites are marked.

ance of unbound protein at 280 nm. The column was washed with 1 M NaCl in TBS, pH 7.4, and equilibrated with washing buffer (TBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM octylglucoside with protease inhibitors). Biotinylated HUVEC extract was loaded on the RGD-α3NC1 column and incubated for 60 min. The column was washed with washing buffer and eluted with 10 mM EDTA in TBS, 50 mM octylglucoside, and protease inhibitors. 0.5-ml fractions were collected, and 20-μl aliquots were tested for the presence of biotin by direct enzyme-linked immunosorbent assay using streptavidin-horseradish peroxidase conjugate (1:10,000; Roche Applied Science). Positive fractions were pooled, supplemented with 20 mM $MgCl_2$, 10 mM $MnCl_2$, and dialyzed against TBS, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.02% NaN_3.

Immunoprecipitation/Western Blotting—Protein A/G-agarose was preabsorbed with unlabeled HUVEC protein extract prepared as described above. Aliquots of dialyzed fraction eluted from RGD-α3NC1 affinity column were preincubated with integrin antibodies in immunoprecipitation buffer (TBS, 1 mM $MgCl_2$, 0.5% Nonidet P-40, 0.1% BSA) for 2 h at 4 °C followed by incubation with protein A/G-agarose beads for 5 h at 4 °C. The beads were washed once with immunoprecipitation buffer and four times with modified RIPA buffer (TBS, 1% Nonidet P-40, 0.5% deoxycholate). Immunoprecipitated proteins were run on 6% SDS-PAGE, transferred to nitrocellulose membranes, incubated with streptavidin-horseradish peroxidase conjugate, and visualized by enhanced chemiluminescence (Pierce).

Solid Phase Ligand Binding Assay—Microtiter plates were coated with various proteins and blocked with 1% BSA/TBS as described for cell adhesion assay. Purified integrins were overlaid in binding buffer (TBS, 0.1% BSA, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, 5 mM octylglucoside) and incubated for 2h at 30 °C. The plates were washed three times with washing buffer (TBS, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.01% Tween 20) and incubated with α_v integrin antibodies (P2W7, 1:500) for 1 h. After extensive washes, the bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse IgG antibodies. *p*-Nitrophenyl phosphate substrate (Sigma) was added to the wells, and absorbance was measured at 410 nm. Nonspecific binding obtained by preincubation of purified integrins with 10 mM EDTA for 30 min at 4 °C was subtracted from all of the obtained values.

Statistical Analysis—The data are expressed as the means ± S.D., and statistical analysis was performed using Student's *t* test for unpaired samples. Differences were considered statistically significant if the *p* values were less than 0.05.

RESULTS

Experimental Strategy and Expression of Recombinant NC1 Domains/Chimeras—In our earlier studies, the human α3NC1 domain was expressed as a recombinant protein containing a 12-residue collagenous sequence at the N terminus, as a strategy to map the locations of epitopes for GP autoantibodies (29, 30). The entire sequence was required to match that of the native fragment, produced by collagenase digestion of native basement membranes, to ensure the preservation of epitopes (31). Subsequently, we used this protein in studies of cell adhesion, migration, tumor growth, and angiogenesis (15). This recombinant protein, equivalent to tumstatin (NCBI accession number AAF72632) in other reports (16, 21), is designated herein as RGD-α3NC1 (Fig. 1*A*) to denote the presence of the RGD site in the short collagenous sequence and to distinguish it from protein containing only the α3NC1 domain. Likewise, α1/α3 chimera, composed of the α1NC1 domain along with the

short collagenous region of α3 chain containing the RGD site, is designated RGD-α1NC1.

In the present study, we examined the functionality of the proximal RGD site and the non-RGD motifs of the RGD-α3NC1 domain in endothelial cell adhesion and integrin binding. To do this, we expressed the α3NC1 with and without the N-terminal RGD sequence and the α1NC1 domain with and without RGD site from α3 chain. Recombinant NC1 domains and chimeras (Fig. 1*A*) were expressed in HEK 293 cells to ensure proper folding and disulfide bond formation, as demonstrated in our previous studies defining conformational epitopes for GP antibodies (29, 30). The migration patterns of purified RGD-α3, RGD-α1, and α3NC1 domains on SDS-PAGE were in agreement with their expected molecular masses (28.4, 28.3, and 27.2 kDa, respectively). Moreover, the RGD-α3NC1 and α3NC1 domains were immunologically identical when checked by Western immunoblotting with conformational-dependent GP and Mab-3 antibodies (Fig. 1*B*), confirming that they were properly folded.

Role of the Proximal RGD Site and NC1 Domain in Endothelial Cell Adhesion to RGD-α3NC1—Our previous data demonstrated differential activity of recombinant NC1 domains of the six α-chains of type IV collagen for endothelial cell interactions (15). In the present study, dose-response curves for cell adhesion to these domains were measured to establish a foundation for subsequent experiments (Fig. 2*A*). Among the NC1 domains, RGD-α3NC1 displays the strongest capacity in promoting HUVEC adhesion and spreading in a concentration-dependent and saturable manner, whereas α1NC1 has minimal effect. The adhesive activity of RGD-α3NC1 was comparable with that of fibronectin (Fig. 2*B*). HUVEC adhesion and spreading on RGD-α3NC1 was completely abolished by preincubation with EDTA (Fig. 2*B*), suggesting that the adhesion is integrin-dependent. Similar results showing preferential cell adhesion to RGD-α3NC1 were observed with three human tumor cell lines: HT-1080, PC-3, and MCF-7 (data not shown).

The presence of the RGD sequence is a unique feature of RGD-α3 when compared with all other NC1 domains. To explore the functionality of the RGD site, as well as the non-RGD motifs within the α3NC1 domain, we constructed recombinant chimeras for gain- and loss-of-function. As shown in Fig. 3*A*, the RGD-α3 and α3NC1 domains were capable of supporting HUVEC adhesion in a dose-dependent and saturable manner; however, the α3NC1 was only 54% as active as RGD-α3NC1. Such a decrease upon the removal of the RGD site from RGD-α3NC1 directly demonstrates the functional role of RGD in cell adhesion. In the case of the α3NC1 domain, which has no RGD site, the remaining cell adhesion is clearly conferred by non-RGD motifs. To further support the role of the RGD site, we used the α1NC1 domain and RGD-α1NC1 chimera (Fig. 3*B*). Although cell adhesion to the α1NC1 domain was minimal, the introduction of a RGD site in RGD-α1NC1 strongly increased its HUVEC adhesion activity to a level higher than α3NC1, albeit not to that of RGD-α3NC1 (Fig. 3*B*). Thus, our results demonstrate the activity of both the RGD site and the non-RGD motifs of the NC1 domain in cell adhesion to RGD-α3NC1 by the gain-of-function (RGD-α1NC1) and loss-of-function (α3NC1) approaches. Moreover, our data indicate that the RGD site plays a dominant role in cell adhesion.

Identity of HUVEC Integrin Receptors for the RGD-α3NC1 Domain—To determine the identity of integrins that may bind to the RGD-α3NC1 and act as mediators of endothelial cell adhesion, we used the direct approach of affinity chromatography. HUVEC membrane proteins were labeled with an impermeable biotin label and solubilized with octylglucoside, and the lysate was applied to a RGD-α3NC1 column. Immunoprecipitation of the EDTA eluate from an affinity column with specific antibodies revealed the presence of $\alpha_v\beta_3$ and smaller amounts of $\alpha_v\beta_5$ integrin heterodimers (Fig. 4*A*). This suggests either a higher affinity of $\alpha_v\beta_3$ integrin toward the RGD-α3NC1 when compared with $\alpha_v\beta_5$, or alternatively, it may result from a somewhat lower expression level of $\alpha_v\beta_5$ integrin (Fig. 4*B*). Molecular masses of integrin heterodimers under nonreducing conditions were about 160 (α_v) and 95 (β_3 and β_5) kDa, which are in agreement with those reported by others (32, 33). With the exception of $\alpha_v\beta_6$, other integrins including $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_5\beta_1$ were also abundantly expressed in endothelial cells (Fig. 4*B*) but were not detected in the fraction eluted with EDTA from affinity column. These results demonstrated a specific interaction between $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and the RGD-α3NC1 domain.

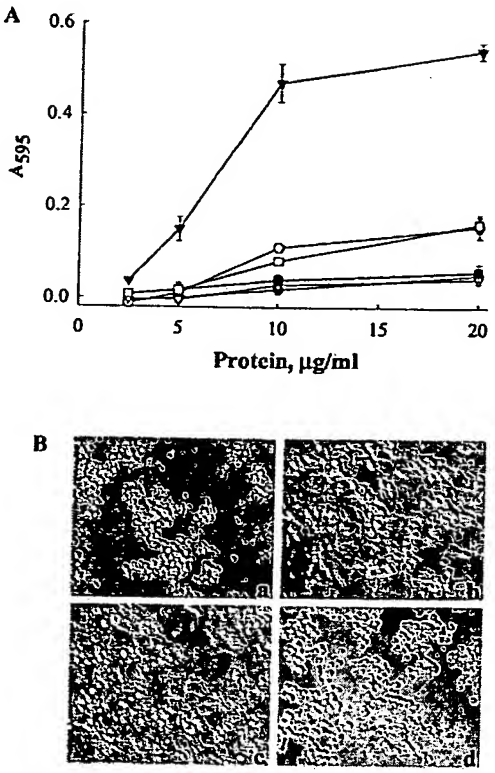

FIG. 2. **Effect of recombinant NC1 domains of type IV collagen on endothelial cell adhesion.** *A*, NC1 domains of type IV collagen (●, α1; ○, α2; ▼, RGD-α3, ▽, α4; ■, α5; □, α6) were coated on 96-well plates, and HUVEC adhesion was determined after incubation for 1 h at 37 °C. Coating efficiency of all NC1 domains determined by enzyme-linked immunosorbent assay with anti-FLAG antibody was essentially equal. The data points represent the mean absorbance ± S.D. of triplicate wells. *B*, phase contrast photographs of HUVEC after incubation for 1 h at 37 °C on wells coated with BSA (*panel a*), 10 μg/ml of fibronectin (*panel b*), or 20 μg/ml of RGD-α3NC1 in the absence (*panel c*) or the presence of 10 mM EDTA (*panel d*) (original magnification 200×). Note the round morphology of nonattached cells in *panels a* and *d*.

Relative Contributions of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrins to Cell Adhesion—To determine the functional significance of $\alpha_v\beta_3$ and $\alpha_v\beta_3$ integrin binding to the RGD-α3NC1 domain in endothelial cell adhesion, we examined the effect of integrin blocking antibodies. Cell adhesion to the RGD-α3NC1, RGD-α1NC1, and α3NC1 domains was strongly inhibited with $\alpha_v\beta_3$ blocking antibodies (Fig. 5*A*). Surprisingly, $\alpha_v\beta_5$ antibodies had no inhibitory effect, either alone or in combination with $\alpha_v\beta_3$ antibodies, suggesting that $\alpha_v\beta_5$ plays a minor role, if any, in endothelial cell adhesion to the RGD-α3NC1 domain. Neutralizing antibodies to α_5 and β_1 integrin subunits blocked HUVEC adhesion to fibronectin and full-length collagen IV, respectively, but had no effect on cell adhesion to RGD-α3NC1, either alone or in com-

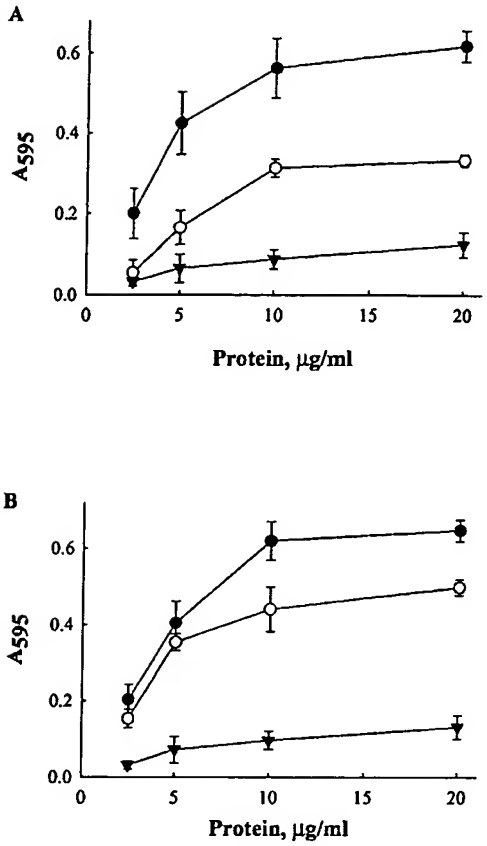

FIG. 3. *A*, HUVEC adhesion to RGD-α3 and α3NC1 domains. The wells were coated with RGD-α3 (●), α3 (○) or α1 (▼) NC1 domains, and adhesion assay was performed as described under "Experimental Procedures." The data points represent the mean absorbance ± S.D. of triplicate wells. This experiment was repeated four times with similar results. *B*, HUVEC adhesion to RGD-α3 and RGD-α1NC1 domains. The wells were coated with RGD-α3 (●), RGD-α1 (○), or α1 (▼) NC1 domains. The data points represent the mean absorbance ± S.D. of triplicate wells. This experiment was repeated five times with similar results.

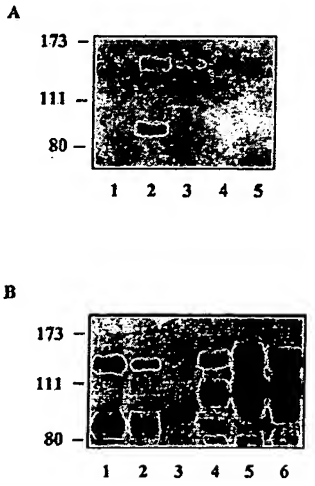

FIG. 4. **Identification of HUVEC integrins bound to the RGD-α3NC1 column.** *A*, immunoprecipitation of the fraction eluted from the RGD-α3NC1 column with EDTA with normal mouse IgG (*lane 1*) or integrin antibodies to $\alpha_v\beta_3$ (*lane 2*), $\alpha_v\beta_5$ (*lane 3*), β_1 (*lane 4*), and α_2 (*lane 5*). Molecular masses of protein markers in kDa are indicated on the *left*. *B*, direct immunoprecipitation of biotinylated HUVEC membrane proteins with integrin antibodies to $\alpha_v\beta_3$ (*lane 1*), $\alpha_v\beta_5$ (*lane 2*), $\alpha_v\beta_6$ (*lane 3*), α_2 (*lane 4*), α_5 (*lane 5*), and β_1 (*lane 6*).

bination with $\alpha_v\beta_3$ antibodies (Fig. 5*B*). Hence, HUVEC adhesion to RGD-α3NC1 is predominately, if not exclusively, mediated by $\alpha_v\beta_3$ integrin.

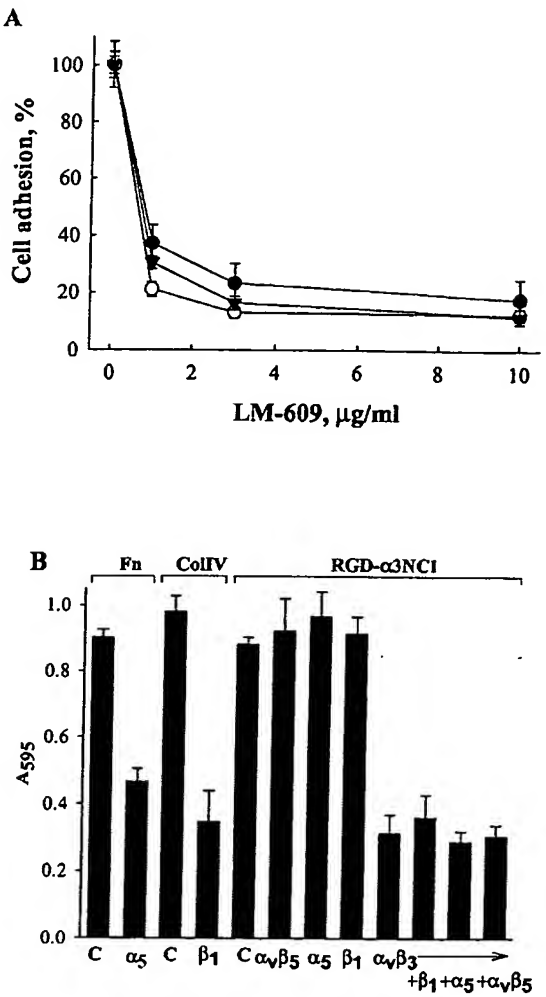

FIG. 5. **Effect of integrin antibodies on the HUVEC adhesion to NC1 domains.** *A*, inhibition of HUVEC adhesion by $\alpha_v\beta_3$ antibodies. The wells were coated with RGD-α3 (●), RGD-α1 (○), or α3NC1 (▼) at 10 μg/ml. Integrin $\alpha_v\beta_3$ neutralizing antibodies (LM-609) were preincubated with cell suspension for 30 min before adding to the wells. *B*, effect of β_1, α_5, and $\alpha_v\beta_5$ antibodies on HUVEC adhesion to RGD-α3NC1. The wells were coated with RGD-α3NC1 (10 μg/ml), fibronectin (3 μg/ml), or type IV collagen from Engelbreth-Holm-Swarm tumor cells (2.5 μg/ml). HUVEC were preincubated without (*lane C*) or with 10 μg/ml of α_5 (BIIG2), β_1 (AIIB2), or $\alpha_v\beta_5$ (P1F6) integrin blocking antibodies alone or in combination with $\alpha_v\beta_3$ (LM-609, 1 μg/ml). The data points/*bars* represent the mean absorbance ± S.D. of triplicate wells. These experiments were repeated three times with similar results.

Contribution of the RGD and Non-RGD Motifs for Binding of Purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrins to RGD-α3NC1—The functionality and relative contribution of RGD and non-RGD motifs of the RGD-α3NC1 domain for binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ was determined by solid phase binding assays using purified integrins. The results show that $\alpha_v\beta_3$ binds to both RGD-α3NC1 and α3NC1 domains in a dose-dependent and saturable manner (Fig. 6*A*). However, the binding capacity of α3NC1 is only 25% of that for the RGD-α3NC1 domain, indicating a strong contribution of the RGD site. Likewise, when the RGD site is attached to the α1NC1 in the RGD-α1NC1 chimera, the $\alpha_v\beta_3$ binding is greatly increased over that of α1NC1 domain (Fig. 6*B*). These results reveal that the RGD site is a major contributor in $\alpha_v\beta_3$ binding to the RGD-α3NC1 domain and that the non-RGD motifs within the α3NC1 domain also contribute to binding, but to a lesser extent. The functionality of the non-RGD motifs is further evident by the greater binding to the α3NC1 domain over that of α1NC1 as well as the RGD-α3NC1 relative to the RGD-α1NC1 domain.

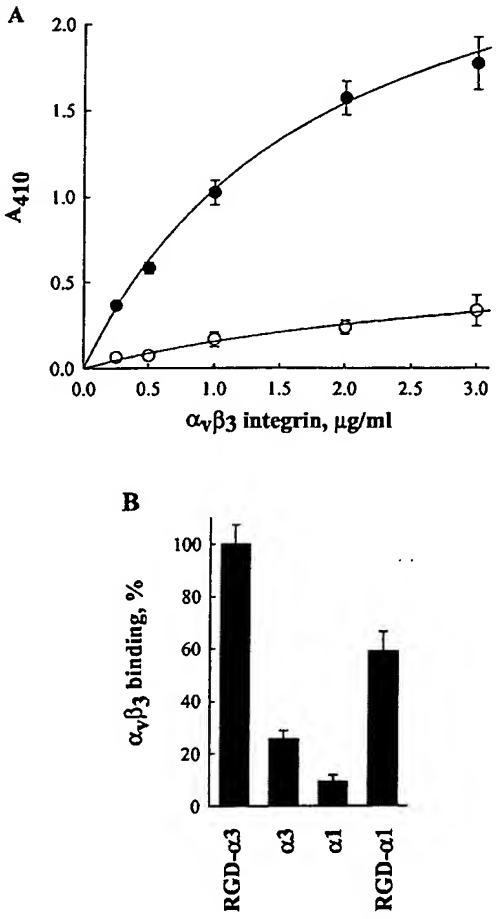

FIG. 6. **Binding of purified $\alpha_v\beta_3$ integrin to immobilized NC1 domains.** *A*, analysis of the $\alpha_v\beta_3$ integrin binding to RGD-α3 (●) and α3 (○) NC1 domains. Immobilized proteins were incubated with purified of $\alpha_v\beta_3$ integrin for 2 h at 30 °C, and bound integrin was detected with α_v-subunit specific antibodies. Specific binding was calculated as the difference of integrin binding without and with 10 mM EDTA. The data represent the means ± S.D. of triplicate wells. The curved lines are the results of hyperbolic nonlinear fitting. This experiment was repeated three times with similar results. *B*, binding of $\alpha_v\beta_3$ integrin to α1 and RGD-α1NC1 domains. $\alpha_v\beta_3$ binding was calculated as the difference of integrin binding to NC1 domains without and with 10 mM EDTA and is expressed as percentages of binding to RGD-α3NC1 domain. The data shown are the means ± S.E. of four independent experiments.

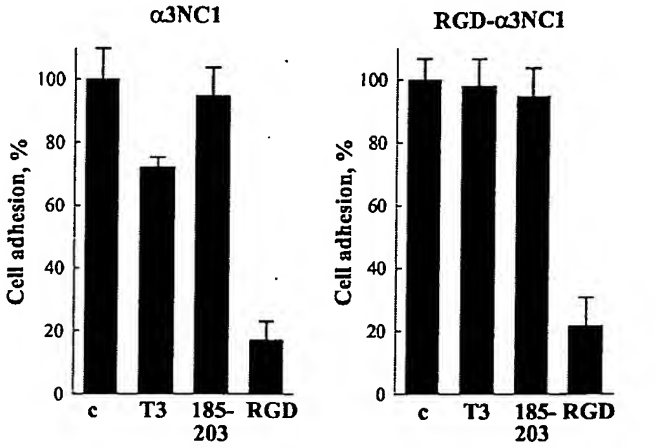

FIG. 7. **Effect of synthetic peptides on HUVEC adhesion to α3NC1 and RGD-α3NC1 domains.** The cells were preincubated with 20 μM of T3, 185–203, or RGD peptides for 30 min and added to wells coated with α3NC1 or RGD-α3NC1 domains (10 μg/ml). HUVEC adhesion was expressed as percentages of maximal binding in the absence of peptides (*lane c*). The data points represent the means ± S.E. of three independent experiments.

In similar experiments with purified $\alpha_v\beta_5$ integrin, the binding to RGD-α3NC1 was significantly lower compared with $\alpha_v\beta_3$ (38.5% averaged from four experiments), despite equal binding of both integrins to vitronectin. Deletion of the RGD site further decreased $\alpha_v\beta_5$ binding by 55% when compared with the RGD-α3NC1 domain, indicating that the RGD motif is a binding site for both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. In addition, no binding was detected of purified $\alpha_1\beta_1$, $\alpha_3\beta_1$, or $\alpha_5\beta_1$ integrins to the RGD-α3NC1 domain under the same conditions (data not shown).

Contribution of the Two Non-RGD Motifs of the α3NC1 Domain in Cell Adhesion and $\alpha_v\beta_3$ Integrin Binding—Utilizing short linear peptides, two RGD-independent sites within the α3NC1 domain have previously been shown to promote adhesion and inhibit proliferation of endothelial and tumor cells. These sites correspond to residues 56–75 (designated peptide T3) and 185–203 of α3NC1 domain (17, 18). Biological activity of both peptides was shown to be dependent on $\alpha_v\beta_3$ integrin binding. Herein, we designate these two integrin-binding sites as non-RGD motifs. Using these two peptides, we addressed whether either or both non-RGD motifs account for full cell adhesive and integrin binding activity of the whole α3NC1 domain (Fig. 1*A*). HUVEC adhesion to the α3NC1 domain was only partially (30%) inhibited by T3 peptide, whereas peptide 185–203 had no effect even at ~100-fold molar excess of soluble peptides over the immobilized α3NC1 domain (Fig. 7). At the same concentration, both peptides had no effect on cell adhesion to the RGD-α3NC1 domain.

The effect of T3 and 185–203 peptides on integrin binding was directly determined using solid phase assay. Binding of the $\alpha_v\beta_3$ integrin to the immobilized α3NC1 domain was competitively inhibited by an excess of soluble α3NC1 with an IC_{50} of ~0.1 μM (Fig. 8*A*). The inhibitory effect of T3 and 185–203 peptides was lower, with an IC_{50} in the low micromolar range. The difference was even more pronounced for $\alpha_v\beta_3$ binding to the RGD-α3NC1 domain (Fig. 8*B*), where both peptides showed only partial inhibition, consistent with the major contribution of the RGD site for binding. Simultaneous addition of both peptides did not cause further inhibition of integrin binding (data not shown). Thus, the inhibition of $\alpha_v\beta_3$ binding to both α3NC1 and RGD-α3NC1 domains by T3 and 185–203 peptides was at least 10-fold less potent than by whole recombinant proteins. Taken together, our data indicate that the two non-RGD peptides do not fully mimic the cell adhesive and integrin binding activity of the α3NC1 domain, from which they are derived.

RGD Peptide Inhibits Cell Adhesion and $\alpha_v\beta_3$ Integrin Binding to RGD-α3NC1 and α3NC1 Domain—Given the fact that the RGD is a potent inhibitor of integrin-mediated cell adhesion to several extracellular matrix proteins, the effect of soluble RGD peptide on cell adhesion and $\alpha_v\beta_3$ integrin binding was measured. RGD peptide at 20 μM strongly inhibited HUVEC adhesion to both α3NC1 and RGD-α3NC1 domains (Fig. 7). Furthermore, cell adhesion to T3 and 185–203 peptides immobilized on solid phase was also strongly inhibited by the soluble RGD peptide (data not shown).

We also used a solid phase ligand binding assay to directly assess whether soluble RGD could inhibit $\alpha_v\beta_3$ binding to the RGD-α3NC1 domain. RGD peptide, at concentrations as low as 0.1 μM, completely abolished the binding of $\alpha_v\beta_3$ integrin to both RGD-α3 and α3NC1 domains (Fig. 9), indicating that integrin binding is significantly more sensitive to the RGD peptide compared with HUVEC adhesion. The inhibition by T3 and 185–203 peptides was at least 400-fold less efficient than RGD, suggesting a higher affinity of $\alpha_v\beta_3$ integrin for RGD compared with non-RGD linear peptides. In addition, we tested

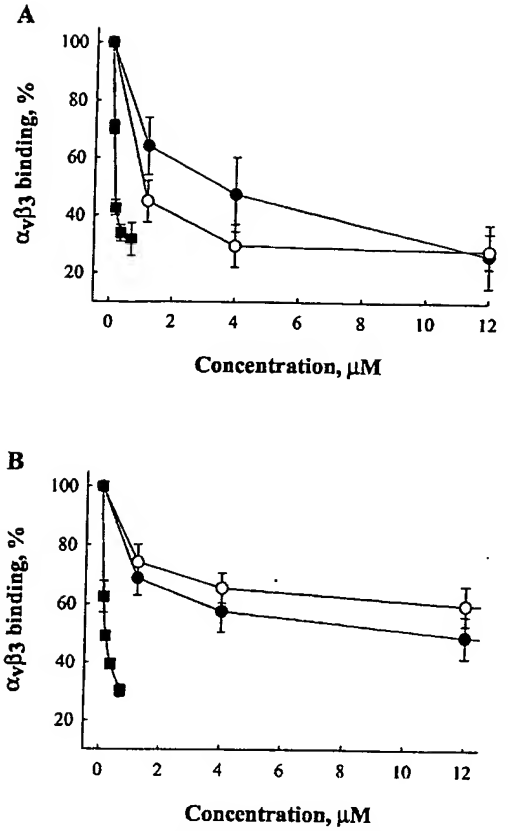

FIG. 8. **Inhibition of $\alpha_v\beta_3$ integrin binding to immobilized α3NC1 and RGD-α3NC1 by soluble NC1 domains and synthetic peptides.** *A*, $\alpha_v\beta_3$ integrin (1 μg/ml) was added to wells coated with α3NC1 domain after preincubation for 30 min with soluble α3NC1 (■), T3 (○), or 185–203 (●) peptides. *B*, the wells were coated with RGD-α3NC1 domain, and $\alpha_v\beta_3$ integrin was added after 30 min of preincubation with soluble RGD-α3NC1 (■), T3 (○), or 185–203 (●) peptides. $\alpha_v\beta_3$ binding was calculated as the difference of integrin binding to NC1 domains without and with 10 mM EDTA and expressed as a percentage of binding in the absence of soluble NC1 domains or peptides. The data shown are the means ± S.E. of four independent experiments.

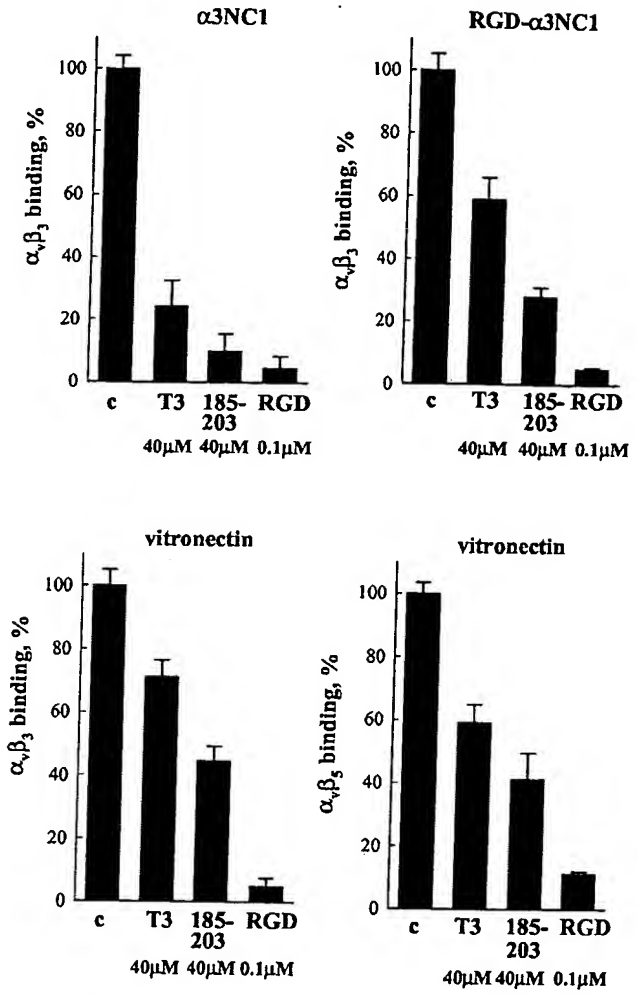

FIG. 9. **Effect of T3, 185–203, and RGD peptides on binding of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins to α3NC1, RGD-α3NC1, and vitronectin.** Purified $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins were added to wells coated with NC1 domains or vitronectin (10 μg/ml) after preincubation for 30 min without any additives (*lane c*) or with T3, 185–203, or RGD peptides. $\alpha_v\beta_3$ binding was calculated as the difference of integrin binding to NC1 domains or vitronectin without and with 10 mM EDTA and is expressed as percentages of binding in the absence of peptides. The bars represent the means ± S.D. of triplicate wells. This experiment was repeated three times with similar results. Note that the concentration of RGD peptide was 400-fold less than T3 or 185–203.

the effect of peptides on binding of purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins to vitronectin, a known RGD-dependent ligand for both integrins (34). As expected, the RGD peptide strongly inhibited binding of integrins to vitronectin. T3 and 185–203 peptides only showed partial inhibition, further supporting the observation that both RGD and non-RGD motifs of RGD-α3NC1 may bind to the same site on $\alpha_v\beta_3$ integrin, albeit with a different affinity. Overall, these results indicate that both the proximal RGD site and the non-RGD motifs of the α3NC1 domain interact with the RGD-binding site on the $\alpha_v\beta_3$ heterodimer.

DISCUSSION

Cell adhesion and the anti-angiogenic activity of RGD-α3NC1 domain has been ascribed to its interaction with $\alpha_v\beta_3$ integrin on endothelial cells (15, 16). In the present study, the functionality of the RGD and non-RGD motifs within the RGD-α3NC1 domain was assessed in the context of native protein conformation. Our results demonstrate a predominant role of the RGD site for endothelial adhesion and for binding of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Moreover, we demonstrate that the two non-RGD peptides, previously identified as the $\alpha_v\beta_3$ integrin-binding sites of the α3NC1 domain, are 10-fold less potent in competing for integrin binding than the native protein, indicating the importance of additional structural and/or conformational features of the α3NC1 domain for integrin binding. Therefore, the RGD site, in addition to non-RGD motifs, may contribute to the mechanisms of endothelial cell adhesion in the human vasculature and the anti-angiogenic activity of the RGD-α3NC1 domain. This finding of a functional RGD site is contrary to a previous report (16); consequently, it impacts the understanding of the mechanism of cell adhesion and anti-angiogenic activity of the RGD-α3NC1 domain.

We demonstrate by the gain- and loss-of-function approaches that the RGD site significantly enhances the inherent capacity of the α3NC1 domain to support endothelial cell adhesion. Contrary to our findings, Maeshima *et al.* (16) reported that this RGD site is nonfunctional, based on the failure of a 20-mer synthetic peptide containing RGD to support adhesion and the lack of inhibition of cell adhesion to the recombinant RGD-α3NC1 domain by the cyclic RGD peptide. This discrepancy may relate to our use of recombinant proteins expressed in HEK-293 cells for native conformation, whereas the RGD-α3NC1 domain expressed in *Escherichia coli* has an unfolded conformation (30), and low coating efficiency or sterical constrains for short synthetic peptides immobilized on solid phase used in their studies.

The cell adhesion to both the RGD-α3NC1 and α3NC1 do-

mains is mediated by $\alpha_v\beta_3$ integrin. This was initially shown for the RGD-α3NC1 domain and its deletion fragments using integrin-blocking antibodies (15, 16). In the present study, the identity of HUVEC integrins that bind the RGD-α3NC1 domain was determined by the direct approach of affinity chromatography. Among the numerous integrins expressed on endothelial cells (35), only $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins bound the RGD-α3NC1 domain in a divalent cation-dependent manner. Moreover, functional studies using blocking antibodies revealed that endothelial cell adhesion to both α3NC1 and RGD-α3NC1 domain is mediated only by the $\alpha_v\beta_3$ integrin.

The binding of $\alpha_v\beta_3$ integrin to the RGD-α3NC1 domain involves interactions with both the proximal RGD site and non-RGD motifs within the α3NC1 domain. In solid phase binding assays, the $\alpha_v\beta_3$ integrin bound to both the RGD-α3NC1 and to α3NC1 domains in a dose-dependent and saturable manner, but the RGD site enhanced the binding by 4-fold. These findings, together with results of adhesion studies, provide strong evidence that (*a*) $\alpha_v\beta_3$ integrin mediates endothelial adhesion to RGD-α3NC1 domain through binding to both RGD and non-RGD motifs and (*b*) the RGD site plays a dominant role in both integrin binding and cell adhesion. The functionality of the RGD site is consistent with numerous reports on its role as a key binding motif for multiple integrins, including $\alpha_v\beta_3$ (26, 36). Contrary to these findings, Maeshima *et al.* (16) found that the RGD site in the RGD-α3NC1 domain (tumstatin) is nonfunctional for binding of $\alpha_v\beta_3$ integrin on the basis of adhesion studies alone, leading them to the conclusion that endothelial adhesion is mediated exclusively by $\alpha_v\beta_3$ binding to non-RGD motifs. This disparity in findings and conclusions may relate to differences in experimental strategies (see above).

The non-RGD motifs that bind $\alpha_v\beta_3$ integrin were previously mapped to two sites within the α3NC1 domain, residues 56–75 and 185–203, with use of short linear peptides (17, 18). These peptides designated T3 and 185–203, supported cell adhesion of endothelial and melanoma cells, respectively. However, as shown in the present study, only T3 had a partial capacity to compete with the whole NC1 domain in cell adhesion assays, and both peptides were 10-fold less potent in competing for the binding of $\alpha_v\beta_3$ integrin in solid phase binding assays. Thus, the two non-RGD peptides do not fully mimic the cell adhesion and integrin binding activities of the parental α3NC1 domain, indicating that the mechanisms of cell adhesion and integrin binding involve additional residues and/or conformational features not present in the linear peptides. It is conceivable that the non-RGD motifs, in the form of short peptides, would not adopt the same β-sheet conformation favorable for integrin binding, as they exist within the context of the native α3NC1 domain (Fig. 1, *C* and *D*). Moreover, they are located at opposite sides of the NC1 domain, suggesting the independent participation of each motif in integrin binding.

It has been proposed that the non-RGD motifs within α3NC1 domain bind to a site on $\alpha_v\beta_3$ integrin distinct from the RGD-binding pocket (16, 23). This suggestion was based on the absence of the effect of RGD peptides on cell adhesion to RGD-α3NC1 domain. Contrary to this finding, however, we found that the soluble RGD peptide strongly inhibits HUVEC adhesion and integrin binding, not only to RGD-α3NC1 but also to the α3NC1 domain. Similar to our results, an inhibitory effect of RGD peptides has been reported for several other $\alpha_v\beta_3$ ligands lacking the RGD sequence, such as the C-terminal fragment of MMP-2 (37), cysteine-rich heparin-binding protein Cyr-61 (38), angiostatin (39), and plasmin (40). Therefore, both the RGD and non-RGD motifs of the RGD-α3NC1 domain may bind to the identical site or spatially overlapping sites on the integrin. Alternatively, these motifs may bind to distinct pockets within the $\alpha_v\beta_3$ heterodimer, which are allosterically interconnected. For example, the existence of two distinct binding pockets has been shown on $\alpha_{IIb}\beta_3$ integrin for the RGD and non-RGD peptides of fibrinogen (41, 42). Moreover, RGD ligands are capable of $\alpha_{IIb}\beta_3$ binding even when it is already occupied by fibrinogen. Thus, if similar binding sites exist within $\alpha_v\beta_3$ for RGD and non-RGD motifs of RGD-α3NC1, they are likely to be mutually dependent as supported by our observation that non-RGD peptides T3 and 185–203 inhibit $\alpha_v\beta_3$ binding to both α3NC1 and the RGD-dependent ligand vitronectin to a similar extent.

The α3 chain of type IV collagen is a major component of the basement membrane that underlies the endothelium of glomerular and alveolar capillaries. Our finding that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins directly interact with α3NC1 domain provides insight into the possible endogenous function of the α3 chain. For example, in the glomerular basement membrane the α3NC1 domain, as a part of the α3·α4·α5 network, could play a role in the attachment of endothelial cells, which express $\alpha_v\beta_3$ integrin (43), contributing to glomerular integrity and ultrafiltration function. However, the accessibility of the non-RGD motifs for $\alpha_v\beta_3$ integrin within the collagen IV network of basement membrane is still unknown. Homology modeling based on the crystal structure of native α1.α2 NC1 hexamer (44) suggests that non-RGD integrin-binding motifs of the α3NC1 domain could be buried within the α3·α4·α5 hexamer and therefore not accessible for binding. It should be noted that among the known mammalian sequences, the RGD site proximal to α3NC1 domain is unique for the human species. Location of this site within the triple-helical domain of collagen molecule makes it a poor candidate for integrin binding. The triple helical domain of α1·α2(IV) collagen, which has 11 different RGD sites, does not bind $\alpha_v\beta$ integrin (45). However, phosphorylation of a serine residue immediately adjacent to RGD sequence observed *in vivo* indicates that the secondary structure of this region could be different from triple helix, suggesting that this RGD site may be accessible to cellular receptors (46).

Our finding that the RGD motif plays a critical role in endothelial cell adhesion strongly suggests that it contributes to the anti-angiogenic or anti-tumor activity of the RGD-α3NC1 domain. This is supported by the capacity of RGD peptides to inhibit angiogenesis and tumor growth (47–49), presumably because of their interference with the adhesion and migration of endothelial cells to extracellular matrix proteins (50). In addition, the RGD site may facilitate targeting of α3NC1 domain to tumor blood vessels, as has been shown for RGD-containing conjugates, such as doxorubicin or monoclonal antibodies (51, 52).

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X. RELATED PROCEEDINGS APPENDIX

None